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14. ABSTRACT

The focus of this program is the development of a pre-clinical blood-based TSE diagnostic assay. The assay in the pilot form is developed with plasma from hamsters infected with the 263K strain of scrapie. The same assay can be adapted to human PrP test. In this funding period we completed the optimization of the conditions for proteinase K (PK) digestion of PrPres in scrapie infected hamster plasma. We have also started a large study to determine the PK concentration that preserves plasma infectivity while reducing the level of endogenous normal PrP to below the limit of detection of our PrP assay. The PK concentration that indicates no infectivity reduction will be used to digest infected plasma for detection of PrPres. We also found that urine excreted by infected hamsters harbors infectivity with infectivity titers similar to that of hamster plasma. More recently, we inoculated animals with urine from pre-clinical hamsters. This study is still on-going and will be completed by the end of the funding period. Urine could be a useful alternative to blood in a TSE diagnostic assay. We also completed the titration of bladder and kidneys with similar titers approximately 5 log10 ID50/g of tissue.

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Introduction

Transmissible spongiform encephalopathy (TSE) diseases are fatal illnesses for which there is no cure or treatment. Individuals incubating TSE can transmit infectivity by blood transfusion. Four human-to-human transmissions of variant Creutzfeldt-Jakob disease were reported in the United Kingdom[1,2]. TSE infectivity in blood was also demonstrated in natural and experimental animal models such hamster[3,4], mouse[5] and sheep[6]. A TSE diagnostic will greatly improve the safety of the blood supply.

Great efforts have been directed towards the development of a pre-mortem preclinical diagnostic test using blood. The landscape of the TSE diagnostics is ever changing, at the present time there are three assays that are gaining momentum: the Prionics test with a PrP^{res} specific monoclonal antibody 15B3[7], the Amorfix assay[8] and the Soto's assay with the protein misfolding cyclic amplification (PMCA) method[9]. The first two assays are capable of detecting brain PrP^{res} spiked in plasma at the same concentration as those estimated to be present in endogenous infected plasma. No human infected blood was assayed. The PMCA assay showed amplification of PrP^{res} from human and animal blood. However, PMCA as a diagnostic assay has come under scrutiny and questioning after the latest report that PrP^{res} signal (and infectivity) can be created from normal PrP^{c} alone[10].

We have been developing a prototype assay for TSE infection using hamsters infected with the 263K stain of scrapie. In our current assay platform, the target test material is plasma and the protein to be detected is PrP^{res}, the biochemical marker for TSE infections. We showed that the assay is capable of detecting abnormal PrP from brain spiked in plasma. In the first attempt to detect PrPres in plasma, no signal was observed after PK. These results highlighted the need to optimize the PK digestion step. This report will focus on the approach we applied to this task.

We also obtained the only quantitative measurements of the titer of infectivity in excreted urine. Three studies were published on TSE infectivity in urine. One study from Gabizon's laboratory showed trace amounts of urinary infectivity in the 236K hamster strain[11]. The second report was from Aguzzi's lab and indicated that only mice with kidney infection and scrapie excreted infectivity in urine. Mice infected with scrapie alone did not have infectivity in their urine[12]. The third study was in deer infected with chronic wasting disease and showed no infectivity in urine and feces inoculated together in 3 deer[13]. The results from our studies were presented at the Prion2006 meeting and elicited strong interest especially on the part of the medical products regulators in Europe. This report will also describe the current status of the urine studies.

Body

In this report as in all previous ones, the specific aims numbering was modified to reflect the elimination of the first specific aim from the original proposal.

Specific aim 1 – Task 1

Urine from clinically infected hamsters and age matched uninoculated control animals was collected in metabolic cages. Clinical and control urine pools were titered using the limiting dilution titration method into weanling hamsters. This protocol was described in the previous report. In brief, we inoculated approximately 5 ml aliquot of the clinical urine pool diluted 1:3 distributed 50 μl per animals into 300 hamsters. Eight animals died shortly post inoculation leaving 292 animals in the study. Similarly, 2 ml aliquot of the urine pool from control animals was inoculated undiluted into 40 hamsters. Inoculations were conducted intracerebrally with the animals under deep anesthesia.

After 559 days post inoculation all animals still alive were euthanized and the brain was removed for detection of the infection-specific PK-resistant PrP^{res} by DELFIA. All clinically scrapie positive animals and those animals that died of intercurrent illnesses during the study were analyzed by Western blot of the brain tissue for the presence of PrP^{res}. There were six animals for which the Western blot could not be resolved either because the PrP^{res} signal was very weak or because it was not reproducible. We inoculated the brain homogenate of these animals into other hamsters (2 animals per case) to make the final determination of the disease status of these animals. The results will be known in three months. Thus the final total number of infected animals may vary slightly from that in Table 1. Table 1 summarizes the results for this study notwithstanding the 6 animals still pending. Eighteen animals inoculated with urine from scrapie infected hamsters developed the disease. The urine infectivity titer measured with the Poisson distribution was 3.8 ± 0.9 ID/ml^4.

We observed one infected animal in the cohort inoculated with normal urine (425 days post inoculation). Following a review of the procedures, we concluded that the most likely cause of this contamination was a technical error at the time when the urine pools were prepared. Two considerations should be emphasized: 1) Urine of control uninoculated animals was not infectious. The control animals did not display scrapie signs and their brains were negative for PrP^{res}. 2) The BSL-3 facility environment is not contaminated. We take extreme care to strictly adhere to the decontamination procedures as demonstrated in many studies in which hundreds of

animals were inoculated with samples that produced no infections for the whole duration of the study (> 540 days). These observations suggest that the single case of transmission in the control urine originated not from the environment or the experimental procedure but from the urine sample that was accidentally contaminated after collection.

Table 1

Sample Urine from	Volume inoculated	dilution	Total animals	infected animals	titer (ID/ml)	SD
Infected hamsters	4.87	3	292	18	3.8	0.9
uninoculated hamsters	2	1	40	1	-	-

Kidney and Bladder titrations

We also titered bladder and kidney tissues from infected animals. Table 2 shows the results of these titrations. The study was terminated at 462 days post inoculation. All animals' clinical scores have been confirmed by Western blot of the brain tissue. The titers calculated with the Reed and Muench method[14] were $10^{5.5+0.5}$ infectious doses$_{50}$ (ID_{50}) per g of bladder and $10^{5.0+0.4}$ ID_{50} per g of kidney. This is the first report of bladder titration and also of a quantitative measurement of the kidney infectivity titer.

Table 2

Dilution	Bladder Tot/Infect	Kidney Tot/Infect
10% (10^{-1})	19/19	4/4
1:2	8/8	20/20
1:5	8/8	8/8
1:10 (10^{-2})	4/4	8/8
10^{-3}	4/4	4/3
10^{-4}	4/2	4/1
10^{-5}	4/1	4/0
10^{-6}	4/0	4/0

The implications of finding infectivity in urine are several and span different aspects of TSE diseases. In the context of a

diagnostic assay urine is a desirable test material because of it can easily obtained in relatively large quantities. Furthermore, the titer of infectivity in urine is similar to that of plasma suggesting that also PrP^{res} concentration may be the same; however, the concentration of protein in urine is about 1000-fold less than that in plasma thus making urine a more suitable test material for diagnostic.

Titration of urine from pre-clinical animals

Following the results of the clinical urine titration, we decided to titer urine collected at the pre-clinical stage of the scrapie infection. At the time we started this study we did not consider the issue that the infectivity titer in urine is affected by the volume of urine produced in a given time. The titration with the standard 5 ml of urine inoculated into 100 animals is still on going but so far (520 days post inoculation) no animals has developed scrapie suggesting that pre-clinical urine is not infectious. This result is unexpected as we anticipated that infectivity in urine as in blood will be present at early incubation times. It is likely that infectivity is below the limit of detection of the bioassay because at pre-clinical stage animals are not dehydrated and the infectivity in urine is diluted in a large volume compared to the clinical urine. Therefore, we would like to repeat the study after a method of urine infectivity concentration is developed.

Specific aim 2 – Task 2

As indicated in our previous report, we have developed a PrP detection system that uses the ORIGEN technology (the instrument is produced by BioVeris, Gaithersburg, MD) to measure PrP in brain and plasma of scrapie infected animals. We have already discussed these results and their implications for a TSE blood-based diagnostics. This task of the proposal has been completed.

We practically have dropped the collaboration with Biotraces as the company was not able to provide us with an improved version of their instrument. Although the claimed sensitivity was reproduced in studies conducted at BioTraces with recombinant PrP. The question was whether the same sensitivity could be obtained with PrP from infected hamster plasma or even brain. This issue was never resolved. We have recently obtained permission from the US Army Medical Research and Material Command to redirect the finding allocated to the BioTrace’s collaboration to continue our studies with urine and PK digestion of plasma.

The issue tackled in the past year was that of incorporation of PK into the assay. As indicated above we attempted to detect PrP^{res} in

hamster infected plasma without success. One of the problems is that the PK digestion was not optimized and therefore it was not possible to know whether PrP^{res} detection failed because of insufficient assay sensitivity or because PK had removed/reduced PrP^{res}. To answer these questions we design the study described in Specific aim 3 tasks 1 and 4.

Specific aim 3 – Tasks 2 and 3

Several issues needed to be addressed before the PK digestion of hamster plasma study could be started. We resolved each issue and the following is a summary of our conclusions.

SDS in PK digestion: Early pilot studies using normal and scrapie brain spikes in plasma indicated that 0.5%-1% SDS was necessary to remove PrP^{c} to below the limit of detection of the assay. This requirement represented a technical problem for the assay and the bioassay. We discovered that SDS was needed only with brain PrP^{c} and not normal plasma PrP^{c}.

PK concentration for plasma PrP^{c} digestion: Once the SDS was removed from the plasma PK digestion, a new optimization of PK concentration was necessary. These studies revealed that high PK concentration (>50 ug/ml) was required for PrP^{c} degradation.

Inactivation of PK before bioassay: The final goal of these studies was to inoculate the PK-treated plasma and measure the infectivity titer. Therefore, the issue of compatibility of the PK-treated plasma samples with the bioassay needed to be addressed. Typically, PK reaction is stopped with PK inhibitors PMSF or pefabloc. Early toxicity studies indicated that both reagents were highly toxic and that the animals tolerated significantly lower concentrations of these inhibitors than it was needed to effectively inhibit PK. This was a difficult problem since active PK, we thought, would also be toxic to the animals and active PK could be a problem during the biochemical assay for PrP^{c} detection (see below). After many tests and toxicity experiments, we concluded that the best option was not to add PK inhibitor in the sample prepared for the bioassay. To our surprise hamsters tolerated high PK concentration intracerebrally inoculated much better than the PK inhibitors. On the other hand, PMSF could be added to the sample dedicated to the detection of PrP^{c} since this was not inoculated into animals.

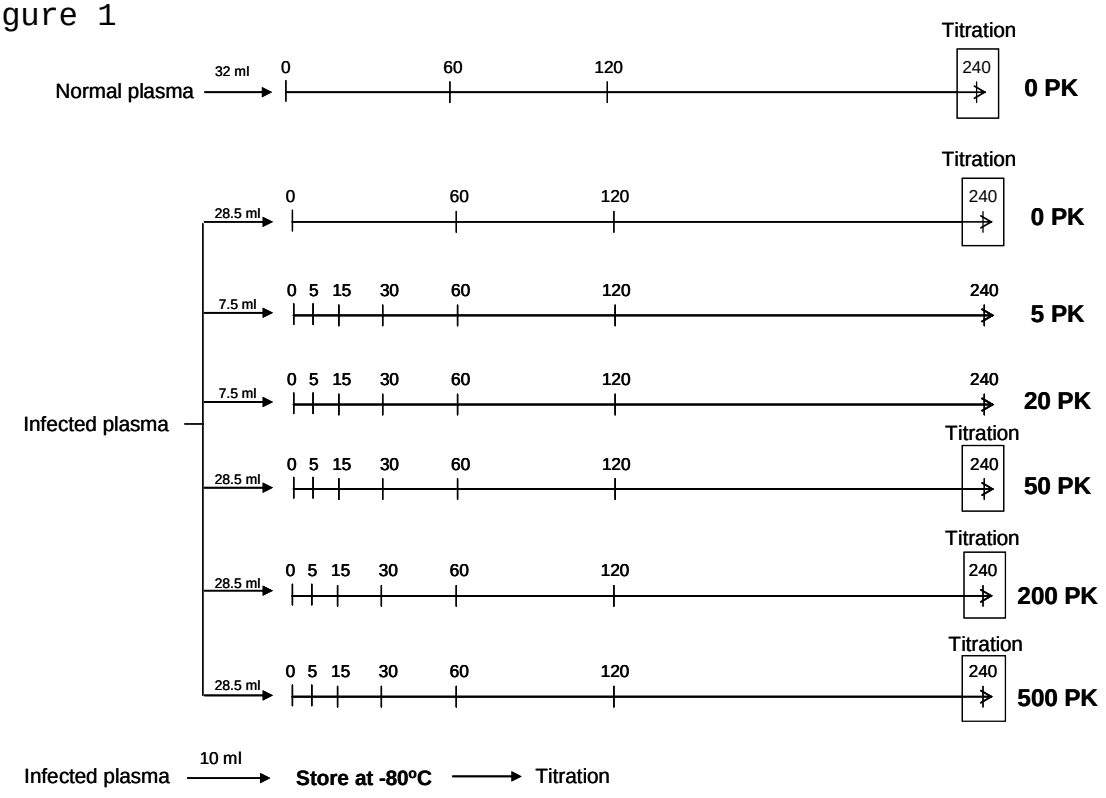
Choice of infected plasma for titration: This was also an issue that set us back several months. We had initially decided to use an infected plasma pool prepared in our laboratory 3-4 years ago. This was the pool used for the pilot tests and it seems at the time a suitable pool to use. However, after long discussions with Dr. Rohwer it was decided that it was too risky to use that plasma pool because infectivity had never been measured and nothing is known about the effect of storage on endogenous plasma infectivity. As a consequence, a fresh pool of infected plasma was produced. 140 hamsters were inoculated intracerebrally with scrapie and 120 days

later they were bled to produce approximately 200 ml of fresh infected plasma. This plasma was used for the study described below.

Specific aim 3 – Tasks 1 and 4

The PK conditions for endogenous plasma PrP^{res} cannot be determined as PrP^{res} cannot be detected. Using the surrogate brain-derived PrP^{res} is not appropriate since the PK conditions are different (see above). Therefore, we proposed to measure the plasma infectivity remaining after PK digestion using the animal bioassay. Assuming correlation between infectivity and PrP^{res}, this study will indicate the appropriate PK conditions to digest PrP^{c} and not PrP^{res}. PrP^{res} is tracked by titration of endogenous infectivity and digestion of PrP^{c} is verified with the ORIGEN assay. To improve the PrP^{c} assay sensitivity an aliquot of the PK-treated plasma was mixed with the 3F4 immuno affinity resin. The resin captures PrP^{c} and improves the assay sensitivity of ~ 10-fold. The study assay design is in Figure 1.

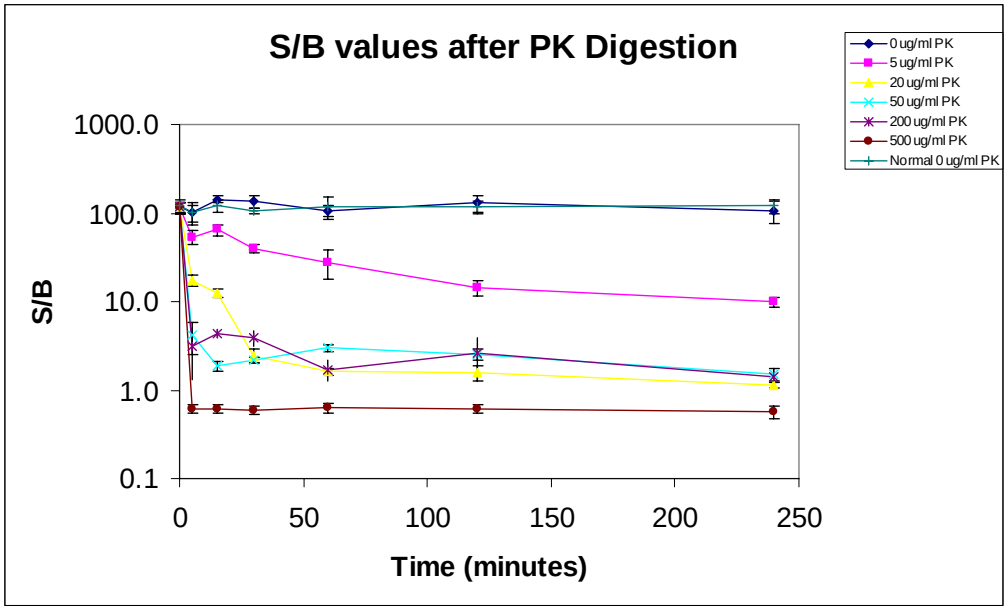
Figure 1



Based on pilot tests, 3 concentrations of PK, 50 ug/ml, 200 ug/ml and 500 ug/ml were selected in addition to no PK as the control. Those concentrations were chosen because they consistently showed digestion of PrP^{c} to below the detection of the assay. The question was under these conditions of PrP^{c} degradation, which PK concentration has no effect on infectivity? Since in this study we

assume that reduction of infectivity implies degradation of PrP^{res}, that condition with no change in the infectivity will be selected aa the assay condition for PrP^{res} detection in plasma. The PK digestion condition on titration was with the reaction incubation at 37^{o}C for 4 hours. A control with normal plasma incubated as the experimental samples was included together with the titration of the plasma pool that had not been incubated. The details of the animal bioassay are below.

Although only five titrations could be conducted we considered analyzing samples at different time points and also to include additional PK concentrations. These samples were only investigated for the concentration of PrP^{c} with the 3F4 capture and the ORIGEN Analyzer detection. The results of this analysis are reported in Figure 2.



The figure represents the log of S/B (signal/background) versus time. The background was calculated using a PrP-depleted plasma sample. S/B = 1 indicates that the sample has a signal equal to that of background. We also found cases in which the signal was below the background (S/B <1), this was interpreted that the PK digestion removed components in plasma that were responsible for the background. Both samples without PK (normal and infected plasma) showed no signal reduction over the 4 hours incubation time. As the concentration of PK increased the digestion curve became steeper and PrP^{c} was digestion quicker. These results are consistent PK digestion of PrP^{c} occurring in a time and concentration dependent. The three conditions titered with the bioassay (50 200, 500 ug/ml) showed an unexpected result. In previous pilot tests, all three conditions indicated PrP^{c}

degradation to the limit of detection of the assay (S/B <1). However in this particular test, only 500 ug/ml PK was below the S/B threshold and 200 ug/ml PK barely reached that threshold at 4 hours time point. 50 ug/ml was slightly above the limit at all time points suggesting that low level of PrP^c may still be present in this sample. If 50 ug/ml of PK is the only condition to show no reduction of infectivity, the interpretation will be that PK is not a suitable method of discrimination between plasma PrP^c and PrP^{res} since the sensitivity of the two proteins to PK is too close to be separated.

Animal Bioassay

Endogenous plasma infectivity can be measured with the limiting dilution method in which 5 ml of sample is distributed into 100 animals (50 ul per animals) by intracerebral inoculation. This method has been used extensively in our laboratory for those samples with extremely low titers such as blood, blood components and urine. Table 3 shows the summary of the various titrations and the number of animals inoculated per each sample. This study will be on going for at least 540 days post inoculation.

Table 3

	Normal plasma	Scrapie plasma				
PK ug/ml	0	0	0	50	200	500
treatment	37oC	-80oC	37oC	37oC	37oC	37oC
Volume assayed (ml)	5.3	5.2	5.4	5.25	5.5	4.5
Animals inoculated	106	104	108	105	110	90

The incubation time is 115 days post inoculation.

Key research accomplishments

- We have measured the infectivity titer of urine excreted by scrapie infected hamsters.
- The pre-clinical urine is still on titration but shows no transmission.
- The measurement of the titers of kidney and bladder from infected hamsters is completed including confirmation with Western blot.
- The titration of PK treated infected hamster plasma was started and is on going.
- We have generated a valuable PK digestion matrix in which

endogenous plasma PrP^c digestion is assayed as a function of time and PK concentration.

Reportable outcomes

Invited presentation at the Prion2006 Meeting in Turin. Title: Infectivity in urine of hamsters infected with scrapie and implications on mechanisms of horizontal transmission.

Invited presentation at the European Medicinal Agency, TSE Expert meeting, London July 2007. Title of the presentation: “Infectivity in urine of hamster infected with scrapie”.

Gregori, L., Rohwer, R.G., 2007. Characterization of scrapie-infected and normal hamster blood as an experimental model for TSE-infected human blood. Dev Biol (Basel) 127, 123-33.

Hamir AH, Kunkle RA, Bulgin M, Rohwer RG, Gregori L, Richt JA. Experimental transmission of scrapie agent to susceptible sheep using intralingual and intracerebral routes of inoculation. The Canadian Journal of Veterinary Research.
Accepted for publication.

Conclusions

The research program was delayed due to issues with the PK digestion and the toxicity of PK inhibitors. Those problems have been addressed and solved. We did not encounter other problems that required revision of the proposed studies. We started a large bioassay study to determine the PK concentration that remove PrP^c to the limit of detection of the biochemical assay and maintains intact the infectivity. Those conditions will be used to digest PrP from large volume of infected plasma for PrP^{res} detection. We have confirmed that all three PK conditions currently under investigation with the bioassay have removed PrP^c. We are now waiting for the results of the bioassay.

Our studies of urine have now conclusively demonstrated that this biological fluid contains low but detectable levels of infectivity and urine is a suitable target for a TSE assay. The current infectivity measurement in the pre-clinical assay is indicating no transmissions. There are issues with the urine dilutions and this result requires further investigations.

References

1. Hewitt PE, Llewelyn CA, Mackenzie J, Will RG. Creutzfeldt-Jakob disease and blood transfusion: results of the UK Transfusion Medicine Epidemiological

Review study. Vox Sang. 2006 91:221-30.

2. http://www.hpa.org.uk/hpa/news/articles/press_releases/2006/060209_cjd.htm

3. Brown P, Rohwer RG, Dunstan BC, MacAuley C, Gajdusek DC, Drohan WN. (1998). The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. *Transfusion* 38:810-6.

4. Gregori L, McCombie N, Palmer D, Birch P, Sowemimo-Coker SO, Giulivi A, Rohwer RG. (2004) Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood. *Lancet* 364:529-31.

5. Cervenakova L, Yakovleva O, McKenzie C, Kolchinsky S, McShane L, Drohan WN, Brown P. (2003) Similar levels of infectivity in the blood of mice infected with human-derived vCJD and GSS strains of transmissible spongiform encephalopathy. *Transfusion* 43:1687-94.

6. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. (2000) Lancet 356:999-1000.

7. Raeber A. (2006) Cambridge Healthtech Institute 10thA forum on TSE diagnostic blood tests. Baltimore, Maryland, 7-9 March 2006.

8. Lehto MT, Peery HE, Cashman NR. Current and future molecular diagnostics for prion diseases. Expert Rev Mol Diagn. 2006 6:597-611.

9. Castilla J., Saá P., Soto C. (2005) Detection of prions in blood. Nat. Med. 11, 982-5.

10. Deleault NR, Harris BT, Rees JR, Supattapone S. Formation of native prions from minimal components in vitro. Proc Natl Acad Sci U S A. 2007 104:9741-6.

11. Kariv-Inbal Z, Ben-Hur T, Grigoriadis NC, Engelstein R, Gabizon R. Urine from scrapie-infected hamsters comprises low levels of prion infectivity. Neurodegener Dis. 2006 3:123-8.

12. Seeger H, Heikenwalder M, Zeller N, Kranich J, Schwarz P, Gaspert A, Seifert B, Miele G, Aguzzi A. Coincident scrapie infection and nephritis lead to urinary prion excretion. Science. 2005 310:324-6.

13. Mathiason CK, Powers JG, Dahmes SJ, Osborn DA, Miller KV, Warren RJ, Mason GL, Hays SA, Hayes-Klug J, Seelig DM, Wild MA, Wolfe LL, Spraker TR, Miller MW, Sigurdson CJ, Telling GC, Hoover EA. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science. 2006 314:133-6.

14. Reed LJ, Muench H. A simple method of estimating fifty per cent end points. Am J Hygiene 1938 27:493-7.

Appendices

None